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Assessing Pathogen Presence in an Intensively Tile Drained, Agricultural Watershed

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Disciplines

Agriculture | Bioresource and Agricultural Engineering | Environmental Monitoring | Water Resource Management

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Assessing Pathogen Presence in an Intensively Tile Drained, Agricultural Watershed

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Abstract

Increases in swine production and concomitant manure application provide beneficial nutrients for crops but also include the potential to spread pathogenic bacteria in the environment. While manure is known to contain a variety of pathogens, little is known regarding the long-term effect of manure application on fate and transport of this diverse set of pathogens into surrounding waterways. We report on the use of 16S-rRNA gene sequencing to detect pathogen-containing genera in the agriculturally dominated South Fork Iowa River watershed, home to approximately 840,000 swine in the 76,000-ha basin. DNA was extracted from monthly grab samples collected from three surface water sites and two main artificial drainage outlets. DNA sequences from water samples were matched with sequences from genera known to contain pathogens using targeted 16S rRNA amplicon sequencing. The specific genera known to contain pathogens were quantified by combining percentage of genera sequence matches with 16S rRNA gene quantitative polymerase chain reaction results. Specifically, abundances of *Bacteroides*, *Clostridium sensu stricto*, and *Acinetobacter* significantly increased in surface water after typical fall manure application. Additionally, the likely transport pathways for specific genera known to contain pathogens were identified. Surface water *Enterobacter* concentrations were influenced mainly by artificial drainage, whereas *Clostridium sensu stricto* was primarily transported to surface waters by runoff events. The results of this study will help us to understand environmental pathways that may be useful for mitigation of the diverse set of pathogenic genera transported in agroecosystems and the capability of manure application to alter existing microbial community structures.

Core Ideas

- *Bacteroides* increased in surface water following typical manure application.
- *Enterobacter* was likely transported to surface water through artificial drainage.
- *Clostridium sensu stricto* was primarily transported to surface water by overland flow.
- *Escherichia* is not a suitable indicator of other genera known to contain pathogens in a water sample.

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INCREASING global pork demands have resulted in growing swine operations and availability of manure for agricultural operations (Galloway et al., 2007). In 2012, Iowa contained over 20 million swine, residing in 6266 farms (NASS, 2014). Manure from swine operations is readily applied to cropland as organic fertilizer. However, such practices would not be practical in parts of north-central Iowa and other parts of the Corn Belt without artificially lowering the water table. Approximately one-third of total cropland in Iowa contains artificial subsurface drainage, commonly referred to as *tile drainage* (Zucker and Brown, 1998). Tile drainage lowers the water table and allows soils that otherwise would be seasonally or continually wet to drain (Zucker and Brown, 1998). This alteration of the hydrologic condition of soil allows for earlier planting and increased soil aeration, which ultimately leads to greater crop yields (Zucker and Brown, 1998; Arabi et al., 2006), but it is also capable of hastening the transport of pollutants, such as pathogens associated with manure. Previous studies identified artificial drainage as a major pathway for nitrogen, dissolved phosphorus, pesticides, antibiotic resistance genes, and other contaminants to connecting surface waters (David et al., 1997; Jaynes et al., 2001; Passeport et al., 2013; Luby et al., 2016). However, less is known regarding the extent of loading and mode of transportation for manure-derived pathogens into surrounding surface waters.

Fecal indicator bacteria (FIB), *Escherichia coli* and enterococci, are commonly used to track the potential transport of manure-associated pathogenic organisms in the environment but have yielded inconsistent results. Significantly higher concentrations of *E. coli* and enterococci in surface water samples have been observed after manure application in the South Fork Iowa River watershed (Givens et al., 2016). In the same watershed, it was also found that *E. coli* concentrations in surface water were greatest during summer months when no manure was applied, suggesting sources other than manure likely influenced *E. coli* loads (Tomer et al., 2008). This result is consistent with other studies that were unable to identify significant differences between enterococcus concentrations in drainage from nonmanured and fall manure-treated plots (Gardner et al., 2014; Hruby et al., 2016; Luby et al., 2016). Overall, these contrasting results

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Abbreviations: FIB, fecal indicator bacteria; GLM, genus-level matching; LOQ, limits of quantification; OTU, operational taxonomic unit; qPCR, quantitative polymerase chain reaction.

suggest that we still do not have a clear understanding of the fate and transport of pathogens at the watershed scale.

One potential reason for contrasting observations is poor correlation between the estimated abundances of specific pathogens in environmental waters, specifically *E. coli* and enterococci, with pathogenic *Salmonella* spp. and *Campylobacter* spp. and *Yersinia* (Lund, 1996; Lemarchand and Lebaron, 2003; Hörman et al., 2004). A meta-analysis of previous studies comparing the correlations of pathogens and *E. coli* abundances resulted in only 26% of 46 data sets showing positive correlations (Wu et al., 2011). Overall, these results suggest that a better understanding of the fate of pathogens in manure requires the integration of data describing more than a single pathogen.

The goal of this study is to examine the persistence of diverse genera known to contain pathogens in surface water and artificial drainage in an agriculturally dominated watershed. These pathogens may originate from widespread manure application within the watershed, which introduces manure-borne pathogens into soils and subsequently into surface water through artificial drainage or overland flow. Additionally, organic matter contained within manure is capable of stimulating naturally occurring genera in soil that contains pathogenic species (Leclercq et al., 2016). We used targeted detection and

community sequencing of the 16S rRNA gene to determine the concentrations of genes associated with genera known to contain pathogens in water samples, allowing us to compare the impacts of timing of manure application and the fate of diverse bacterial genera known to contain pathogens in waters downstream of agricultural practices on microbial community structure.

Materials and Methods

Study Site and Sample Collection

Our study site is the 76,000-ha South Fork Iowa River watershed, located mainly in Hardin and Hamilton Counties in central Iowa. The watershed is dominated by corn (*Zea mays* L.)–soybean [*Glycine max* (L.) Merr.] row crop agriculture (Supplemental Fig. S1). The watershed contains 169 confined animal feeding operations, which are estimated to provide swine manure to 30 to 60% of the watershed at a rate of 93 to 186 m³ ha⁻¹ (Givens et al., 2016). Analysis of 2008 to 2009 land cover and confined animal feeding operation location and size in the South Fork Iowa River Watershed indicated that approximately 43 ± 5% of the watershed received manure application during the 2-yr span (Fig. 1). Detailed descriptions of sampling

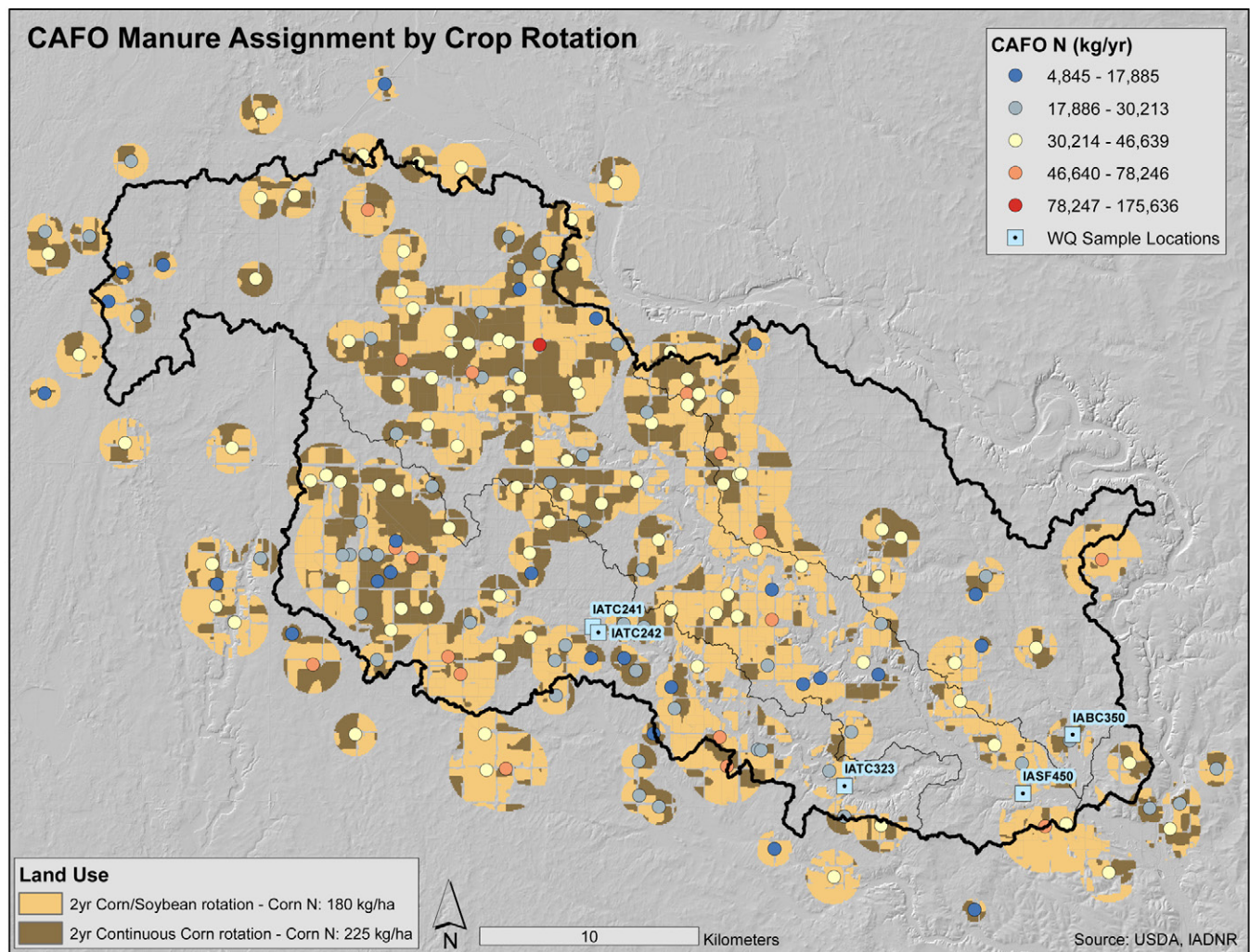


Figure 1: Predicted manure application rates developed from analysis of swine populations based on confinement size, estimated manure and manure N content, and application of manure assuming minimal transport and crop rotation derived from NASS imagery. CAFO, confined animal feeding operation; WQ, water quality.

locations and collection procedures were previously described (Rieke et al., 2018). Briefly, grab samples were collected from August 2011 through December 2014 approximately monthly from three surface locations and two artificial subsurface drainage outlets in the South Fork Iowa River watershed. This resulted in collection of 69 drainage samples and 118 surface water samples. The three surface sampling sites were located on Tipton Creek (TC323), Beaver Creek (BC350), and the South Fork Iowa River (SF450); the two artificial drainage locations (TC241 and TC242) feed directly into Tipton Creek (Fig. 2). Additionally, Tipton Creek feeds into the South Fork main branch upstream of site SF450. Land use of all subwatersheds used in this study contains similar landscapes and is highly representative of the watershed as a whole (Fig. 2, Supplemental Fig. S1).

DNA Extraction and 16S-rRNA Gene Quantitative Polymerase Chain Reaction Quantification

Sterile 0.22- μ m filters (Millipore) were used to filter water samples (250–500 mL) within 24 h of collection. Filters were immediately frozen at -20°C for DNA extraction at a later date using Mo Bio Power Water DNA kits. Primer sequences, quantitative standards, and amplification conditions (primers Eub338/Eub518) to detect 16S rRNA bacterial genes in water samples were previously described (Luby et al., 2016). Methods for setting limits of quantification (LOQ) for these assays were also previously described (Rieke et al., 2018).

16S rRNA Gene Sequencing and Analysis

Extracted DNA was sequenced by amplifying the V4 region of the bacterial 16S rRNA gene as described by Kozich et al. (2013). Raw sequences were deposited in the Sequence Read Archive under SRA accession SRP154941 (SRX4450839–SRX4451025). Amplicon sequences were analyzed using Mothur v.1.37.0 (Schloss et al., 2009). Specifically, chimeras de novo were removed with Usearch (8.1 64 bit) (Edgar et al., 2011). Ribosomal Database Project's (RDP's) 16S rRNA gene database (No. 15) was used to remove chimeras of known reference genes. Unique operational taxonomic units (OTUs) and their abundances in each sample were derived from chimera filtered high quality sequences based on 97% sequence dissimilarity by CD-HIT (4.6.1) (Fu et al., 2012). CD-HIT was used because of its speed and demonstrated production of clusters that were highly similar to true numbers of OTUs derived from simulated complex data (Bonder et al., 2012; Chen et al., 2013). Taxonomy was assigned to each representative OTU sequence using RDP classifier (Wang et al., 2007) with a confidence cutoff at 50% ($-c$ 0.5). Operational taxonomic units observed fewer than five times cumulatively were removed from the dataset. Additionally, samples containing fewer than 12,000 sequences were removed, which resulted in 187 samples. Remaining samples were rarified to 12,462 sequences using the function `rarify_even_depth` in the R package `phyloseq` (McMurdie et al., 2013). Analysis of variance using Bray Curtis distance matrices was performed with the *adonis* function found in the R package `vegan` on OTUs representative of

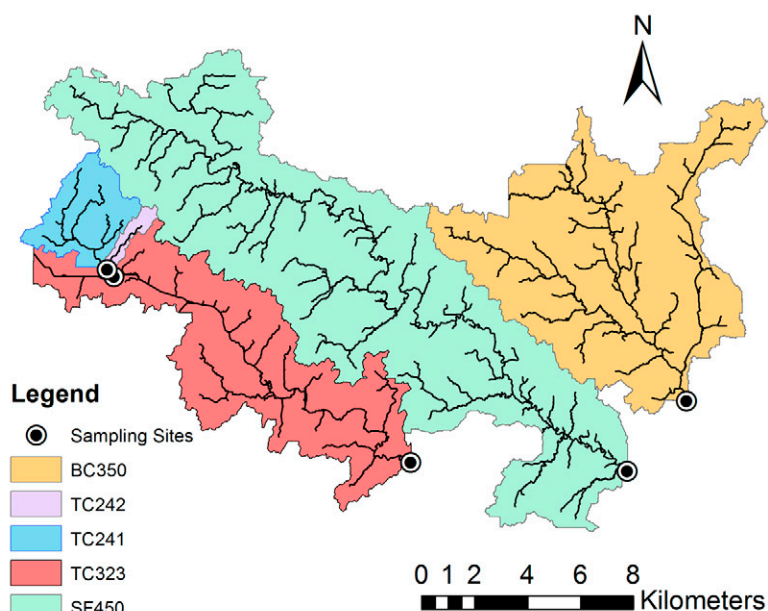


Figure 2: Subwatershed drainage areas and sample locations.

genera containing pathogens to determine which variables contributed to significant differences in the dataset.

Abundance Estimation of Pathogens

Total 16S rRNA genes (copies 100 mL⁻¹) were determined by quantitative polymerase chain reaction (qPCR) assays. To determine the number of specific genera in each water sample, OTUs were classified by genera as described above to estimate the proportion of total number of sequences with genus-level matching (GLM) relative to total number of 16S rRNA sequences (% GLM, or proportion of community with genus-level matching). The total abundance of GLM OTUs was subsequently calculated as the product of total 16S rRNA gene 100 mL⁻¹ and percentage GLM, resulting in total GLM gene copies 100 mL⁻¹. Previous studies used a similar approach for estimating total genus abundance in environmental samples (Ye and Zhang, 2011; Ibekwe et al., 2013, 2018). The total GLM copies 100 mL⁻¹ for each water sample were compared using the Wilcoxon Ranked Sum tests using R version 3.3.1, with samples with significant differences in drainage types and temporal groupings identified as $p < 0.05$. Detailed explanation of sample temporal groupings was described by Rieke et al. (2018). Briefly, samples were grouped into four categories based on soil temperature and typical drainage patterns: frozen soil (~December–March), drainage period (April–mid-July), pre-fall manure (mid-July–September), and post-fall manure (October–November). Analysis of variance was performed in R using the *adonis* function to detect differences in temporal groupings of flow between sampling years.

Results

Pathogens in Subsurface and Drainage Waters of the South Fork Watershed

Overall, we observed significantly different abundances of genes associated with genera known to contain pathogens in varying drainage types ($p < 0.05$) and in different seasons ($p < 0.05$). Genes associated with these genera in individual

sampling locations within the same drainage grouping were not significantly different. Therefore, the three surface water sites and two artificial drainage sites were combined for further analysis. We analyzed the presence of genes associated with six genera previously described as containing manure-borne pathogens: *Bacteroides*, *Clostridium sensu stricto*, *Enterobacter*, *Escherichia/Shigella*, *Serratia*, and *Yersinia*. Genus-level matching *Clostridium sensu stricto* genes were highly prevalent in drainage and surface waters (Table 1), followed by GLM *Enterobacter* genes in drainage water, with 81% of samples containing GLM sequences. However, GLM sequences associated with *Yersinia* and *Bacteroides* were more prevalent in surface water than *Enterobacter*. Genus-level matching *Escherichia/Shigella* and *Serratia* sequences were the least prevalent of the six manure-borne genera. Genus-level matching *Escherichia/Shigella* OTUs were only identified in 27% of drainage samples and 7% of surface water samples, while only 16.7% of surface water samples and 8.9% of drainage samples contained GLM *Serratia* sequences.

In addition to the potential for manure-borne pathogen transport, widespread manure application in the watershed also introduces nutrient rich organic matter. Several studies reported increased proportions of *Gammaproteobacteria* following incorporation of inorganic fertilization, organic amendments or treated wastewater additions (Cleveland et al., 2007; Jangid et al., 2008; Langenheder and Prosser, 2008; Philippot et al., 2009; Ceja-Navarro et al., 2010; Frenk et al., 2014). Sequences associated with three genera within the *Gammaproteobacteria* class containing known pathogenic species were found in the majority of drainage and surface water samples. Specifically, GLM *Legionella* and *Pseudomonas* genes were identified in 100% of drainage samples, while GLM *Acinetobacter* genes were detected in 95.5% of drainage samples (Table 1). Over 90% of

surface water samples were positive for GLM *Legionella* and *Pseudomonas* genes.

Average Daily Flow Analysis

Average daily flow of the three surface water sites and two drainage sample locations varied greatly over the course of the experiment (Table 2). However, no significant differences ($p > 0.05$) between interannual flow temporal grouping were observed. The greatest average daily flows for both surface water and artificial drainage were observed during the drainage period temporal grouping. Surface water average daily flows during the drainage period ranged from $>10^{-2}$ to $>10^3 \text{ m}^3 \text{ s}^{-1}$. The impact of artificial drainage on surface water flows is observed in the log scale increases of total discharge in surface water during the drainage period temporal grouping (Fig. 3). Additionally, the influence of artificial drainage or surface water is observed in similar average daily flow trends of TC241 and TC242, which both flow into Tipton Creek before TC323, and average daily flow in TC323. Furthermore, previous work in the watershed identified similar concentrations of agriculturally relevant antibiotics sulfamethazine and tylosin at sampling locations TC241 and TC323 (Washington et al., 2018). Linear regression yielded significant correlations between the majority of log-transformed 16S rRNA copies 100 mL^{-1} of pathogen containing genera and average daily flow linear regression by site (Supplemental Table S1). Significant correlations yielded r values ranging between 0.44 and 0.68.

Pathogen Temporal Trends and Modes of Transport

Significant temporal differences in *Bacteroides* GLM copies 100 mL^{-1} were observed in both surface water and tile drainage samples (Fig. 4). *Bacteroides* GLM copies 100 mL^{-1} in surface

Table 1. Detection frequencies and concentrations of genes matching genera containing pathogenic species (GLM) in surface water and tile drainage.

Representative genus†	Genus source	Genus source reference	Artificial subsurface drainage		Surface water	
			% of samples containing GLM > LOQ‡	GLM mean (>LOQ) \pm SD	% of samples containing GLM > LOQ‡	GLM (>LOQ) \pm SD
			%	16S-rRNA copy 100 mL^{-1}	%	16S-rRNA copy 100 mL^{-1}
<i>Pseudomonas</i>	widespread in the environment	Vos et al., 2009	100	$1.13 \times 10^6 \pm 3.73 \times 10^6 \text{ a}\S$	95.5	$7.87 \times 10^5 \pm 2.62 \times 10^6 \text{ a}$
<i>Legionella</i>	fresh water environments	Fields et al., 2002	100	$7.48 \times 10^4 \pm 1.52 \times 10^5 \text{ a}$	91.1	$1.35 \times 10^5 \pm 5.04 \times 10^5 \text{ a}$
<i>Acinetobacter</i>	soil, surface water, sewage, food	Bergogne-Bé et al., 1996	95.5	$1.14 \times 10^6 \pm 3.43 \times 10^6 \text{ a}$	78.6	$9.14 \times 10^5 \pm 3.86 \times 10^6 \text{ a}$
<i>Clostridium sensu stricto</i>	human and animal gastrointestinal tracts, water, soil	Cabral, 2010	89.4	$4.12 \times 10^4 \pm 1.11 \times 10^5 \text{ a}$	94.6	$1.12 \times 10^5 \pm 2.53 \times 10^5 \text{ b}$
<i>Enterobacter</i>	skin, water, soil, human and animal gastrointestinal tracts	Cabral, 2010	81.8	$9.79 \times 10^4 \pm 3.49 \times 10^5 \text{ a}$	58.9	$5.61 \times 10^4 \pm 2.00 \times 10^5 \text{ b}$
<i>Yersinia</i>	surface water, soil, feces	Yang Tan et al., 2011	78.8	$4.79 \times 10^4 \pm 1.39 \times 10^5 \text{ a}$	74.1	$6.39 \times 10^4 \pm 1.80 \times 10^5 \text{ a}$
<i>Bacteroides</i>	human and animal gastrointestinal tracts	Cabral, 2010	72.7	$1.21 \times 10^5 \pm 3.66 \times 10^5 \text{ a}$	79.5	$4.39 \times 10^4 \pm 1.26 \times 10^5 \text{ a}$
<i>Escherichia/Shigella</i>	human and animal gastrointestinal tracts	Cabral, 2010	27.3	$3.38 \times 10^3 \pm 6.13 \times 10^3 \text{ a}$	7.1	$5.95 \times 10^3 \pm 5.82 \times 10^3 \text{ a}$
<i>Serratia</i>	water, plants, small mammals	Leclerc et al., 2001	16.7	$4.52 \times 10^3 \pm 1.04 \times 10^4 \text{ a}$	8.9	$1.72 \times 10^4 \pm 3.34 \times 10^4 \text{ a}$

† Operational taxonomic units whose sequences were 97% similar to genera using the Ribosomal Database Project classifier tool (Wang et al., 2007)

‡ Percentage of samples above limit of detection. GLM, genus-level matching; LOQ, limits of quantification.

§ Different letters represent significantly different concentrations of genera in surface water and drainage samples.

Table 2. Average daily flow ranges and total discharge for surface water and artificial drainage, categorized by temporal groupings.

Site	Temporal grouping	Average daily flow range	Total discharge
		$\text{m}^3 \text{s}^{-1}$	m^3
BC350	Frozen soil	3.11×10^{-3} – 4.26×10^1	1.48×10^7
	Drainage period	3.65×10^{-2} – 8.37×10^1	1.40×10^8
	Pre-fall manure	1.95×10^{-2} –9.96	9.85×10^6
	Post-fall manure	2.61×10^{-2} –1.10	2.54×10^6
SF450	Frozen soil	1.16×10^{-2} – 3.57×10^1	1.79×10^7
	Drainage period	1.35×10^{-1} – 1.82×10^2	3.27×10^8
	Pre-fall manure	2.55×10^{-3} –8.24	2.12×10^7
	Post-fall manure	7.63×10^{-3} –4.16	4.28×10^7
TC323	Frozen soil	1.13×10^{-3} – 1.46×10^1	9.39×10^6
	Drainage period	4.16×10^{-2} – 5.03×10^1	9.82×10^7
	Pre-fall manure	4.25×10^{-3} –2.98	5.25×10^6
	Post-fall manure	6.23×10^{-3} –1.05	1.17×10^6
TC241	Frozen soil	0 – 9.96×10^{-2}	6.86×10^4
	Drainage period	5.66×10^{-4} – 8.8×10^{-1}	2.10×10^6
	Pre-fall manure	0 – 4.53×10^{-3}	6.09×10^3
	Post-fall manure	0 – 1.13×10^{-3}	1.20×10^3
TC242	Frozen soil	0 – 8.16×10^{-2}	5.18×10^4
	Drainage period	2.83×10^{-4} – 1.27×10^{-1}	6.83×10^5
	Pre-fall manure	0 – 2.44×10^{-2}	4.91×10^4
	Post-fall manure	0 – 1.36×10^{-2}	7.54×10^4

waters were significantly less in pre-fall manure samples than the three other temporal classifications ($p < 0.05$). In tile drainage waters, *Bacteroides* GLM copies 100 mL^{-1} were significantly greater during the drainage period than both pre-fall manure and post-fall manure periods ($p < 0.05$). We did not observe significant differences in *Bacteroides* GLM copies 100 mL^{-1} in tile drainage samples collected before and after manure application, suggesting the genus was not immediately transported through drainage following typical manure application timing. However, in surface waters, *Bacteroides* concentrations were likely affected by overland flow following manure application where post-fall manure surface water concentrations were significantly greater than pre-fall manure surface water concentrations and tile drainage concentrations did not significantly increase ($p > 0.05$) following the pre-fall manure temporal classification.

Significant temporal trends in *Clostridium sensu stricto* GLM copies 100 mL^{-1} were also identified in tile drainage and surface waters (Fig. 5). Tile drainage GLM copies 100 mL^{-1} followed the same pattern as *Bacteroides*, with significantly greater

concentrations during the drainage than both pre-fall manure and post-fall manure sampling periods ($p < 0.05$). However, surface water concentrations did not follow similar trends. *Clostridium sensu stricto* GLM copies 100 mL^{-1} were significantly greater in surface water during the frozen soil time period than the other three temporal classifications ($p < 0.05$). Additionally, surface water GLM copies 100 mL^{-1} during the post-fall manure period were significantly greater than pre-fall manure GLM copies 100 mL^{-1} ($p < 0.05$). Significantly greater *Clostridium sensu stricto* GLM copies 100 mL^{-1} in surface waters compared with drainage waters (Table 1) likely indicates that the genus was not primarily transported to surface water through subsurface drainage waters. Additionally, significant increases in surface water concentrations following the pre-fall manure temporal grouping suggest *Clostridium sensu stricto* GLM copies 100 mL^{-1} in surface waters were stimulated by manure application in the watershed.

Unlike other genera examined, *Enterobacter* GLM copies 100 mL^{-1} in drainage waters did not significantly differ between temporal classifications. Additionally, *Enterobacter*

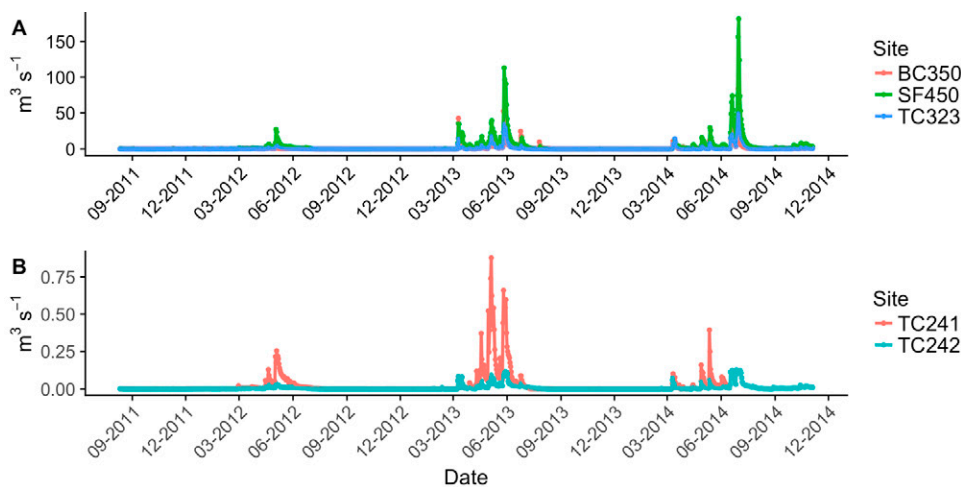


Fig. 3. (A) Average daily flow of surface water over time in South Fork of the Iowa River. (B) Average daily flow of tile drainage over time.

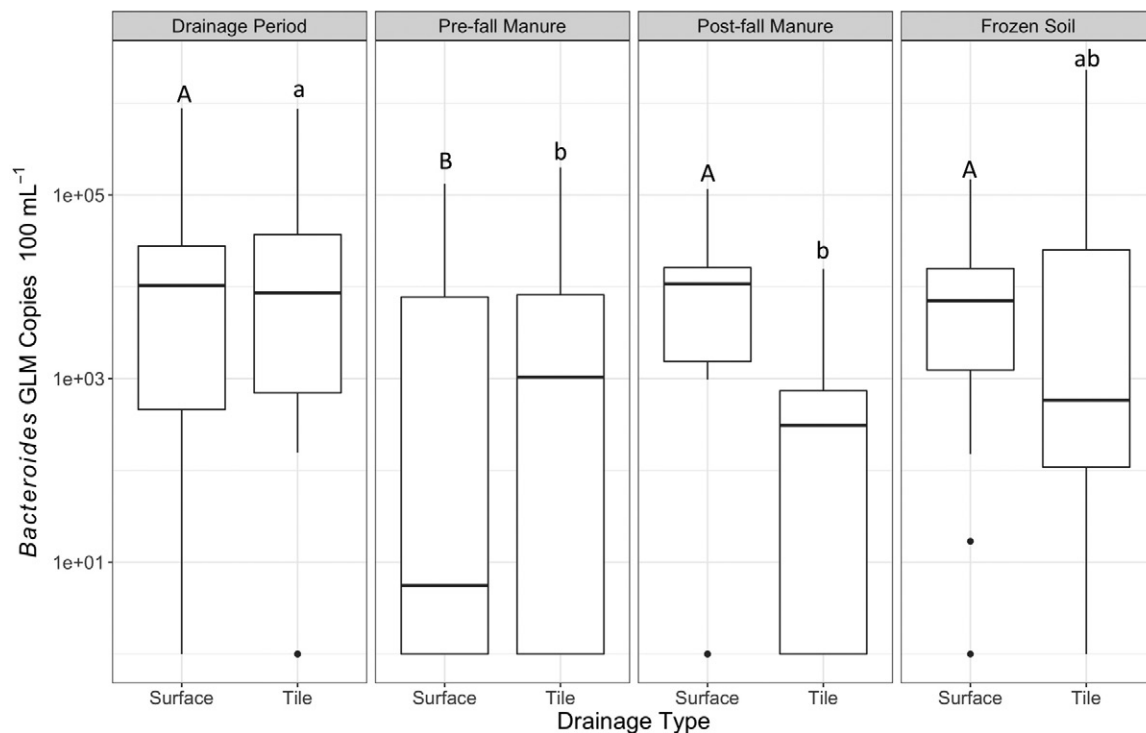


Fig. 4. Concentrations of *Bacteroides* (genus-level matching [GLM] copies 100 mL⁻¹) in surface water and artificial drainage water by season. Samples with *Bacteroides* below limits of quantification are represented as a value of 1. Significantly different surface water gene concentrations ($p < 0.05$) are denoted by different uppercase letters. Significantly different tile water gene concentrations ($p < 0.05$) are denoted by different lowercase letters. The center bar in the box represents the median and the top of the box represents 75th percentile, while the bottom of the box represents the 25th percentile. Whiskers represent minimum and maximum values and dots represent outliers, which are either three times the interquartile range or more above the 75th percentile or three times the interquartile rate or more below the 25th percentile.

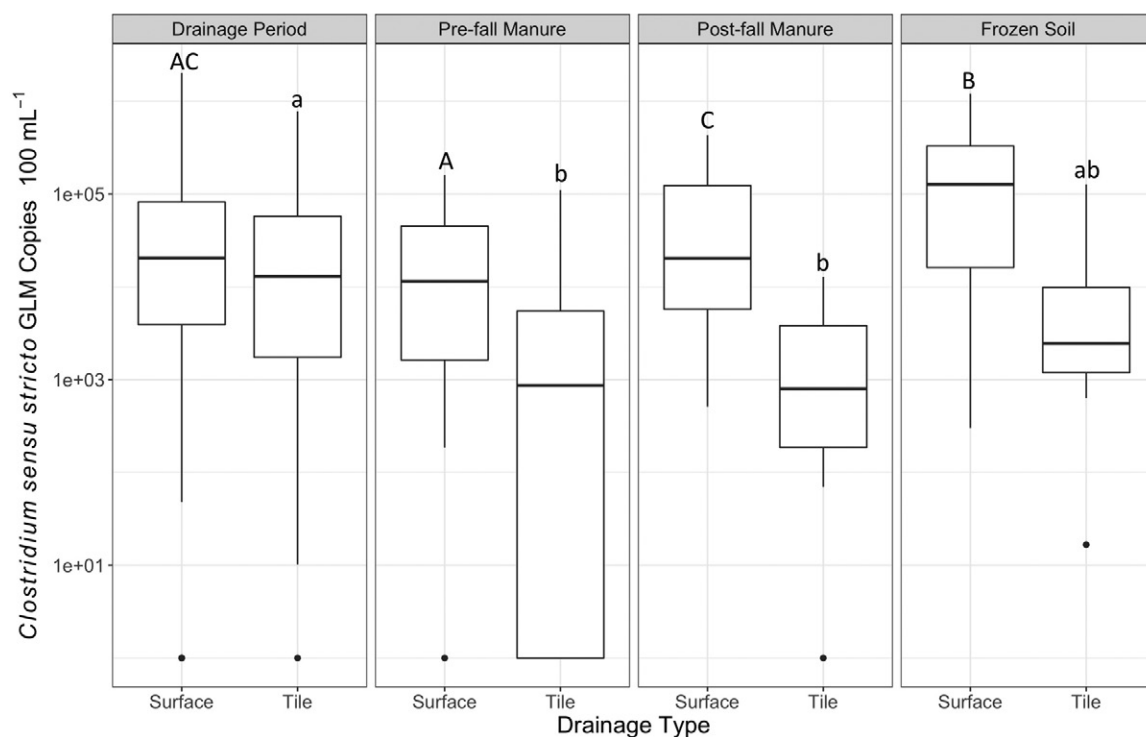


Fig. 5. Concentrations of *Clostridium sensu stricto* (genus-level matching [GLM] copies 100 mL⁻¹) in surface water and artificial drainage water by season. Samples with *Clostridium sensu stricto* below limits of quantification are represented as a value of 1. Significantly different surface water gene concentrations ($p < 0.05$) are denoted by different uppercase letters. Significantly different tile water gene concentrations ($p < 0.05$) are denoted by different lowercase letters. The center bar in the box represents the median and the top of the box represents 75th percentile, while the bottom of the box represents the 25th percentile. Whiskers represent minimum and maximum values and dots represent outliers, which are either three times the interquartile range or more above the 75th percentile or three times the interquartile rate or more below the 25th percentile.

GLM copies 100 mL^{-1} were only significantly greater in surface water in the post-fall manure period when compared to the frozen soil period ($p < 0.05$). However, *Enterobacter* GLM copies 100 mL^{-1} were significantly greater in drainage than surface waters ($p < 0.05$), indicating drainage as the main mode of transport for the genera into surface water. Similar to *Enterobacter*, *Yersinia* GLM copies 100 mL^{-1} revealed no significant difference in drainage waters in the different sampling periods. However, the genus was significantly greater during the drainage period than pre-fall manure in surface waters ($p < 0.05$). Additionally, post-fall manure surface waters were only significantly greater than frozen soil ($p < 0.05$). The lack of temporal trends and greater concentrations identified in surface water may indicate sources other than farm animal manure being responsible for concentrations found in surface waters.

Acinetobacter GLM copies 100 mL^{-1} in drainage samples were not directly affected by typical manure application timing as post-fall manure samples were not significantly different than pre-fall manure and frozen soil time period samples (Fig. 6). However, surface water samples were likely influenced by overland runoff following typical manure application timing as significant increases were noted in post-fall manure samples when compared to pre-fall manure samples ($p < 0.05$) and nonsignificant differences were identified in tile drainage during the sample time periods. Surface water *Acinetobacter* concentrations collected during post-fall manure precipitation events had an average of 1.66×10^8 copies 100 mL^{-1} , while the nonevent average concentration was 2.62×10^7 100 mL^{-1} . Conversely, insignificant increases noted after typical fall manure in surface water suggest that *Legionella* and *Pseudomonas* populations were not directly

affected by the addition of manure organics to soils within the watershed. Additionally, concentrations of both genera in drainage were significantly greater during the drainage period and pre-fall manure periods when compared to post-fall and frozen soil period. Furthermore, concentrations of *Pseudomonas* in drainage samples were significantly greater ($p < 0.05$) during the months of April through September when compared to rest of the year, indicating increased abundance and movement through soil during periods of higher temperatures, where average monthly highs ranged from 27 to 33°C.

Discussion

Combining phylogenetic marker sequencing and qPCR assays, we were able to estimate the abundance of genes associated with genera known to contain pathogens in water samples. This approach allowed us to expand beyond traditional FIBs for the surveillance of multiple pathogens. Our results indicate that different genera have varying modes of transport; however, concentrations of all nine genera analyzed significantly correlated with average daily flow ($p < 0.05$). Significantly greater concentrations of *Enterobacter* in tile drainage waters suggest artificial drainage as a major pathway for this genus to reach surface waters. In contrast, *Clostridium sensu stricto* concentrations were significantly greater in surface waters, indicating overland flow as the major mode of transportation into surface water. These results are consistent with previous studies showing that different bacteria have various mechanisms of attachment to soil and sediment particles (Pachepsky et al., 2006). Similar concentrations in both tile drainage and surface waters were observed in

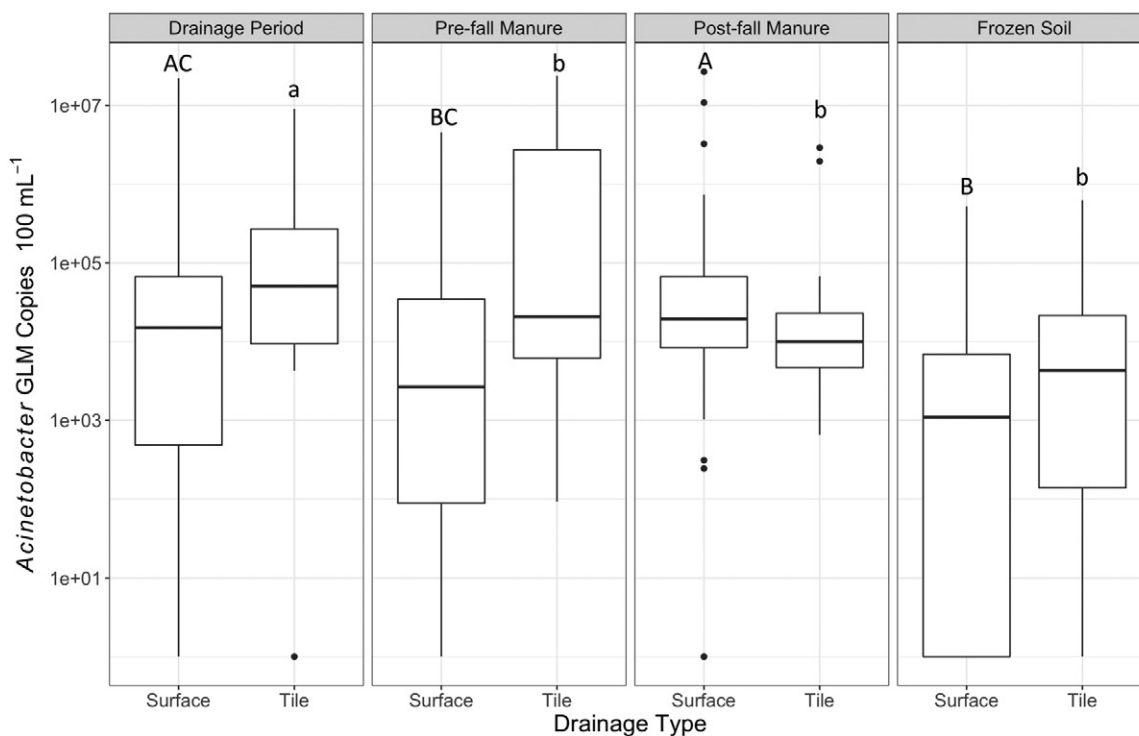


Fig. 6. Concentrations of *Acinetobacter* (genus-level matching [GLM] copies 100 mL^{-1}) in surface water and artificial drainage water by season. Samples with *Acinetobacter* below limits of quantification are represented as a value of 1. Significantly different surface water gene concentrations ($p < 0.05$) are denoted by different uppercase letters. Significantly different tile water gene concentrations ($p < 0.05$) are denoted by different lowercase letters. The center bar in the box represents the median and the top of the box represents 75th percentile, while the bottom of the box represents the 25th percentile. Whiskers represent minimum and maximum values and dots represent outliers, which are either three times the interquartile range or more above the 75th percentile or three times the interquartile rate or more below the 25th percentile.

the genes associated with the remaining seven studied genera, suggesting a sizable impact of drainage on surface waters within the watershed. Previous work estimated artificial drainage was responsible for 71% of discharge from the watershed (Green et al., 2006).

Results from this study also emphasize the effects that fall manure application and seasonal variations may have on bacterial loads in surface waters in an agricultural watershed. Seasonal trends varied between the genera associated with manure-borne pathogens, which were present in the majority of surface and drainage waters. *Clostridium sensu stricto* and *Bacteroides* concentrations in surface water both increased after manure application. These results are consistent with previous studies identifying strong correlations between these genera and swine fecal sources (Dick et al., 2004; Mwaikono et al., 2016). In our water samples, the lack of seasonal variation in *Enterobacter* and *Yersinia* concentrations also indicates manure application does not affect surface water loading of these genera.

In addition to directly increasing certain bacteria through manure additions, long-term incorporation of carbon sources has proved to increase specific bacteria at lower taxonomic classifications (Marschner et al., 2003; Zhong et al., 2010; Cederlund et al., 2014). Furthermore, incorporation of organic fertilizer has shown to have a greater impact on microbial community structure when compared to changes following inorganic additions (Li et al., 2017). Specifically, previous studies attributed increases of *Gammaproteobacteria* to nutrient-rich additions (Cleveland et al., 2007; Jangid et al., 2008; Langenheder and Prosser, 2008; Philippot et al., 2009; Ceja-Navarro et al., 2010; Frenk et al., 2014), and our results suggest shifts of certain genera within the class are also stimulated by temperature fluctuations. Leclercq et al. (2016) attributed increases in relative proportions in *Pseudomonas* spp. and *Acinetobacter* spp. to the addition of manure in soil microcosms incubated at 25°C. We observed surface water concentrations of genes associated with *Acinetobacter* to also be significantly greater after fall manure additions, which occur after soil temperatures drop below 10°C. However, concentrations of gene sequences associated with *Pseudomonas* in surface water did not significantly increase after manure application. Additionally, concentrations of *Pseudomonas* in drainage samples were significantly greater during the months of April through September compared with the rest of the year, indicating increased abundance and movement through soil during periods where average monthly high air temperatures ranged from 27 to 33°C.

Differences in detection limits must be taken into account when comparing studies using different methods to enumerate the same target organism. In this study, we used a combination of genotypic methods (sequencing and qPCR assays), resulting in LOQs greater than traditional FIB methods. In the current study, the LOQ for a specific GLM OTU is dependent on the number of sequences observed per sample and the total number of 16S rRNA gene copies 100 mL⁻¹ per sample, resulting in LOQs ranging from approximately 10² copies 100 mL⁻¹ to >10³ copies 100 mL⁻¹. These LOQs generally fall outside the acceptable range of USEPA protocols for enumerating *E. coli* in water by membrane filtration as colony-forming unit counts between 20 and 80 per sample (USEPA, 2009). However, in general, molecular techniques are increasingly used for pathogen surveillance, evidenced

in the 2012 version of Recreational Water Quality Criteria document, which expanded to include empirical equations capable of relating *E. coli* qPCR data to membrane filtration results (USEPA, 2012).

Importantly, we observed that the presence of *Escherichia* in a sample was not a suitable indicator for the presence of other genera containing pathogens in a water sample, highlighting the need to understand the limitations of FIB assays for interpreting water quality. Current microbial water quality standards implemented by the USEPA are based on empirical dose–response relationships for *E. coli* and enterococci (USEPA, 2012). The methods use colony-forming unit or qPCR FIB data to predict the number of highly credible gastrointestinal illnesses (HCGI) per 1000 contact recreators (USEPA, 2012). Utilization of standards associated with FIB provide insight into the risk of acquiring a gastrointestinal illness but cannot provide information on the causative agent or how detrimental its virulence factor may be to an individual. Varying modes of transport and temporal trends of pathogenic genera reported in our study further suggest the need to better understand the potential for manure application to introduce pathogens into surrounding environments. Improvements in pathogen DNA sequence databases and DNA sequencing technology may provide improved sensitivity and greater application of these methods to water quality assessment.

Supplemental Material

Supplemental material includes: correlation analysis of 16S rRNA sequences of bacterial genera containing pathogenic species and stream or drainage flow, a map depicting land cover and confined animal feeding operation locations in the South Fork Iowa River watershed, and figures depicting bacterial genera containing pathogenic species over time.

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